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TITLE: [ ] PROGRESS REPORT [ ] PHASE REPORT [x] ANNUAL REPORT [ ] TERMINATION REPORT

Spore Formation and Spore Germination of Anaerobic Food Spoilage Organisms, Especially Clostridium Botulinum.

SUMMARY

A simple reasonably accurate method for quantitative study of spore germination in Clostridium botulinum and other anaerobes is described. The obstacle of dormancy has been eliminated, maximum counts appearing in three days. Illustrations are given of the application of the method.

*This report is one of four, to be numbered 4(I), 4(II), 4(III), and 4(IV). The four reports will comprise the annual report on this project.

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Physiological studies on spore germination, with special reference to Clostridium botulinum*

I. Development of a Quantitative Method

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By far the majority of studies hitherto made on germination of bacterial spores have employed the appearance of visible turbidity as the criterion of germination. Obviously this technique can reveal no quantitative characteristics of the germination process and is therefore of value only in establishing that some germination does or does not take place. Even here its value may be questioned, since it has been clearly demonstrated that germination of spores of various organisms may occur without significant subsequent vegetative proliferation. (Itano and Neill, 1919; Knight and Fildes, 1930; Knaysi, 1945; Knaysi and Baker, 1947). Furthermore, various environmental conditions imposed upon germinating spores may have no influence on the germination time but yet may alter appreciably the rate of subsequent vegetative development (Evans and Curran, 1943). Our own experiments confirm this finding.

Direct microscopic counts have been used for quantitative studies of the germination of aerobic spores, (Eckelmann, 1918; Curran, 1931) but such a procedure is unduly wearisome and not readily adapted to use with anerobes. Also,

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with certain species, for example _Bacillus_ _anthracis_, it may be very difficult to establish microscopically a criterion of germination, as noted by Fischoeder (1909), Swann (1927), and Cook (1932).

The outstanding physiological difference between spores and vegetative cells of any one organism, namely, heat lability of the latter at a temperature innocuous to the former, has long been employed in quantitative approaches to spore germination, since it is assumed traditionally that when a spore cell is so changed that it becomes heat labile, germination has taken place (Weil, 1901; Fischoeder, 1909; Evans and Curran, 1943). Although Curran and Evans (1937, 1945b) have indicated that the heat-labile state may actually precede rupture of the spore wall and that some morphological changes characteristic of germination may occur prior to the loss of thermal resistance of the spore, heat differentiation of the germinated vs. the ungerminated spore appears to be the most practicable approach. The fact that a definite reproducible standard endpoint may be selected, viz., survival at a definite temperature for a definite period of time, even though somewhat arbitrary, outweighs the overlapping between the physiological and morphological characters which renders the germination process an indistinct one.

Consideration of other possible criteria of germination have repeatedly brought us to the conclusion that changes in heat lability is best from every point of view, and it forms the basis of this work. The technique has been designed especially for _Clostridium_ _botulinum_, an important organism in food poisoning, on which apparently no quantitative germination studies of any sort have been made. The method has been applied, with appropriate modification, to other anaerobic species.

Experimental

Some of the preliminary work was carried out with _C_. _botulinum_ Strain 115 B;

Strain 62 A was utilized in most of the work. Both strains were obtained from the National Canner's Association and were repurified by isolation of colonies from serial shake tubes. Toxin formation was demonstrated for Strain 62 A by the fact that 1.0 ml. of a Seitz filtrate of a 10-day broth culture was lethal for a guinea pig in less than 21 hours, while a control animal receiving 1 ml. of the filtrate inactivated at 80 degrees C. for 15 minutes survived the observation period of three weeks.

Spore suspensions were prepared from 15 day cultures in Difco brain heart infusion broth with BBL thioglycollate supplement added. After four washings the cells were heated to 75 degrees C. for 30 minutes to destroy vegetative forms and then diluted in sterile distilled water containing glass beads. The final suspensions were then shaken one hour on a rotary shaking machine to break up clumps and stored in the refrigerator. The efficacy of the homogenation procedure was shown by repeated comparisons of counts by plating procedures and direct microscopic counts using the Petroff-Hauser chamber. The former averaged about 50% of the latter, which may be considered fairly good correlation.

Other anaerobes used were C. chauvei, C. histolyticum, C. perfringens, and the well-known food spoilage organism designated as putrefactive anaerobe no. 3679.

The dormancy problem.

The phenomenon of dormancy or delayed germination has presented a formidable experimental difficulty which doubtless has been largely responsible for the lack of any really quantitative studies on spore germination in C. botulinum. Thus various authors report germination occurring only after incubation periods ranging from 53 days to 5 1/2 years! (Burke, 1919, 1923; Starin, 1924; Weiss, 1921; Sommer, 1930; Dozier, 1924; Dickson, et. al. 1922, 1925; Esty and Meyer,

1922; Dickson, 1928). Dormancy is not restricted to C. botulinum spores. It has been established for spores of other clostridia (McCoy and Hastings, 1928), for spores of aerobic species (Burke et. al., 1925; Magoon, 1926; Morrison and Rettger, 1930a and 1930b), and even for cells of Bact. coli (Burke, et. al., 1925). Vegetative cells of C. botulinum have been observed to exhibit a degree of dormancy roughly comparable to that shown by the spores (Starin, 1924).

The prime requisite for systematic quantitative germination studies on "bot" spores is the complete elimination of the dormancy which has handicapped virtually all previous studies with this organism in this connection. Acting on the belief that cultural environment probably conditions dormancy, we felt that the germination medium offered the best prospects for our objective. This had been shown to be the case for aerobic spore formers in which dormancy could be eliminated by supplying the correct medium (Morrison and Rettger, 1930a and 1930b; Curran and Evans, 1937). In recent years improvements have been made in media which now give much higher counts than was possible with media formerly used and which indicates a high degree of success in eliminating the extremely long incubation period during which the spore count as measured by colony development would continue to increase.

Pork infusion[1]· thioglycollate medium has been found to give considerably higher counts on a given spore suspension than other media ordinarily used for

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1. The following procedure for preparation of this medium was kindly furnish by Dr. J. Yesair of National Canner's Association:

One pound of finely chopped lean pork is added to a liter of distilled water and boiled one hour. After removal of the meat and fat, the filtrate is adjusted to pH 7.4 and to each liter is added 5 gms. of peptone, 1.6 gms. tryptone, 1. gm. dextrose, 1.25 gms. K_2HPO_4, and 15 gms. of agar.

It is our practice to omit the dextrose and add 5 gms. BBL thioglycollate

such studies, but no indication is available that maximum counts are obtained in short enough time to be a useful tool for routine studies (Brewer, 1940; Williams and Reed, 1942). Even this medium could be enhanced remarkably (30 fold) in the total count of heated spores obtainable after 21 days by the addition of 0.1 percent soluble starch directly to the germination medium (Olsen and Scott, 1946).

Our work confirms both the superiority of pork infusion as a germination medium and the striking accentuation of starch on the germination process, and consequently on the spore counts. We have further demonstrated that starch acts primarily to adsorb and thus render inactive small amounts of substances present in all media which repress spore germination. This study is the subject of a separate paper. Finally, the spore counting procedure ultimately evolved seemingly has eliminated dormancy as a practical obstacle in quantitative germinative studies on "bot" spores and furthermore for the first time enables maximum counts to be obtained in an incubation period no longer than that required for sizable colony development of any anaerobe, namely 3 days.

Comparison of several media popularly employed for counting C. botulinum spores.

One spore suspension heated to 75 degrees C. for 30 minutes to destroy vegetative cells was serially diluted in triplicate in the various agar media in flat Prickett counting tubes and incubated at 37 degrees C. An effective anaerobic seal was obtained by covering the solidified agar with 3 to 4 ml. of 2 percent agar containing BBL thioglycollate supplement. Prickett tubes are essentially flattened test tubes and, though not generally used, have the decided advantage of permitting colony counts to be made in a thin layer of agar

1. (cont'd.) supplement and 1 gm. soluble starch per liter. We also adjust the final medium to pH 7.4. The troublesome precipitate resulting on boiling the final medium may be removed by filtration under negative pressure or discarded after decanting the supernatant.

instead of the entire diameter of a test tube. If maximum spore counts obtained in the pork-thioglycollate-starch medium are represented as 100, pork medium without starch gave 75, Difco brain-heart infusion 55-60, brain-heart plus 25% peptone (cf. Bristol 1925) 55, BBL anaerobic agar in Brewer anaerobic dishes as employed by Curran and Evans (1946) 28, Difco liver-veal 20 and Wilson and Blair's (1925) agar 5. Not only were counts consistently maximum in pork medium with thioglycollate-starch supplement but they occurred much earlier, reaching the peak in 4 days' incubation as compared to about 3 weeks for brain-heart infusion agar. Actually counts could be made on the second day but the colonies at this point are really too small to count easily or accurately. Three-day-old colonies provide no difficulties. If dormancy exists at all in the pork-starch medium it is believed to be a negligible interference in quantitative studies. Counts, i.e., colonies originating from germinating spores with C. botulinum have never been observed to increase appreciably on prolonged incubation up to 2-3 weeks whereas with other media the results are meaningless before that time. Counts of spore suspensions have regularly been around 50+ percent of direct microscopic counts (Petroff-Hauser chamber), a not too unsatisfactory correlation considering the tendency of the spores to clump in various degrees, and the fact that viability doubtless is not 100 percent. A special experiment to detect dormant spores (i.e., ungerminated viable spores) in this medium after 3 days' incubation failed to reveal any that germinated up to time of present writing, a period of 4 1/2 months. Typical dormancy under these conditions manifests itself as gradually increasing counts over the entire incubation period.

<u>Detailed procedure for studying germination of C. botulinum spores.</u>

The following procedures typify our approach to the quantitative study of the germination process and of factors influencing it. One ml. portions of the appropriately diluted spore suspension were transferred to tubes containing

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9 ml. of Difco brain heart infusion broth containing BBL thioglycollate supplement. This particular germination medium was chosen because the relatively moderate rate of germination occurring in it allows the study of factors both stimulatory and inhibitory to germination. The tubes were then heated to 75 degrees for 20 minutes to expel dissolved oxygen and to effect any possible "heat activation" of the spores (cf. Evans and Curran, 1943; Curran and Evans, 1945a). After appropriate intervals of incubation at 37 degrees C. in air or other atmosphere, replicate (usually triplicate) tubes were re-heated to 75 degrees C. for 20 minutes to destroy any vegetative cells which had developed as a result of germination. Residual spore counts were made as above in pork-thioglycollate-starch agar. Available data indicate that germination is somewhat faster at 30 degrees than at 37 degrees; but for convenience 37 degrees was used in all these germination studies.

Expression of results.

Most workers have utilized absolute numbers of residual spores as a basis for interpreting the effect of a particular treatment on the germination process. We feel that percentage germination is to be preferred as a more reliable basis for interpretation of results, because of the large populations employed, and especially is this true when germination is largely complete. For example, on the comparative basis of residual relatively small spore counts, Evans and Curran (1943) concluded that a considerable acceleration of germination of aerobic spores had resulted from pre-heating the spores in glucose broth. If, however, the residual spores are considered as a fraction of a large population and calculated as percentage of that population, the stimulatory effect for 4 out of the 7 positive cases would be less than 6 percent and in one instance less than 0.1 percent. Certainly the magnitude of the effect

is much different when expressed percentage-wise, the only valid way, in our estimation. A simple numerical example of this point seems worthwhile. Suppose a germination test is run under 2 treatments on a spore suspension containing 5,000 spores per ml. and residual spore count shows 100 and 200 per ml. respectively. While the 100 percent difference between the residual spore counts seems striking, the values for <u>germinated</u> spores are the design of the experiment and the more important data. These would be 4,900 and 4,800 respectively, or 98 and 96 percent germination, an insignificant difference in work of this nature.

<u>Application to germination under stimulatory and under inhibitory treatments.</u>

Though germination curves may be employed for determining the effect of a given factor throughout the time course of germination, the effect taken at any one significant incubation time is usually sufficient.

If a stimulatory factor is being studied, the time selected should be such that germination is relatively small in the control in order to allow the treatment to manifest itself to the maximum. An example is the effect of 0.1% soluble starch in the germination medium (brain heart broth) shown in Table I. The spore counting medium was the usual pork-thioglycollate-starch agar.

On the other hand, an inhibitory effect is best demonstrated at an incubation time when germination is nearly maximum in the controls. Table 2 demonstrates that germination in brain heart broth is considerably retarded by momentary contact with air during removal of sample tubes for counting from a desiccator made anaerobic with an inert gas phase (natural gas, CH_4), even though re-exhaustion with a Hyvac pump and replacement with inert gas is done without delay. In the unopened desiccator 87 percent of the spores germinated whereas in the desiccator opened briefly at 20 and 24 hours only 29 percent germination

was obtained -- a striking inhibition.

Accuracy and reproducibility of spore counts.

The degree of accuracy obtainable with the above method depends, of course, on the number of replicates used for determining the "average" counts. For zero controls triplicate tubes were generally used, with triplicate dilution plated for each tube, or a total of nine counts. For other averages, triplicate tubes with duplicate or triplicate platings of dilutions were usually employed. The overall reproducibility and accuracy of counts on a C. botulinum spore suspension stored in the refrigerator is illustrated in Table 3.

Agreement between replicate counting tubes seems to depend on several factors, including scrupulous chemical cleanliness of glassware, the presence of soluble starch in the counting medium and the atmosphere in which germination takes place.

Factors conducive to variability.

Considerable evidence has been accumulated that germination of "bot" spores is extremely susceptible to minute amounts of substances in the general category of impurities. As mentioned earlier these occur in all organic media and possibly in tap water. At any rate, a high order of variation was experienced between replicate tube counts of a given dilution of the suspension until a rigorous cleaning procedure was adopted. A marked reduction in count variation followed when the cleaning was done with "Dreft", followed by thorough rinsing with distilled water. However, the best means of minimizing this tube to tube variation proved to be the additional feature of incorporation of the starch. The adsorption effect mentioned above explains this levelling action of the starch.

Finally, even though the cultivation of the germination tubes in an atmos-

phere of ordinary air gives good growth, use of an inert atmosphere of natural gas further reduced appreciably the count variation in replicate tubes.

The above procedures work equally well with the four other anaerobic spore-formers tested.

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Table 1

EFFECT OF STARCH ON GERMINATION

Starch	Incubation, hours	Av. Count Residual Spores	Germinated Spores	% Germination
-	0	575	-	-
-	24	400	175	30
0.1%	24	60	515	90

Table 2

EFFECT OF MOMENTARY CONTACT WITH AIR ON GERMINATION IN NATURAL GAS

	Incubation hours	Av. Count Residual spores	Germinated spores	Percent germination
Desiccator unopened	0	560	--	--
	23	74	485	87
Desiccator opened twice to remove samples at 20 and 24 hours	0	535	--	--
	28	380	155	29

Table 3

REPRODUCIBILITY OF SPORE COUNTS ON A SINGLE SUSPENSION

Date of Count	Spores per ml.
3/10	535
3/15	560
3/27	540
4/2	575
4/9	530
4/20	560
4/20	520
4/24	600
4/27	500
4/30	535
5/22	540
6/1	580
Average	548

Extreme deviation from mean: $\frac{50}{548} = 9\%$

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TITLE: [ ] PROGRESS REPORT [ ] PHASE REPORT [x] ANNUAL REPORT [ ] TERMINATION REPORT

Spore Formation and Spore Germination of Anaerobic Food Spoilage Organisms, Especially Clostridium Botulinum.

SUMMARY

The germination process of spores of **C.** botulinum 62A is logarithmic. In air atmosphere the length of the lag period in germination varied inversely with the logarithm of the number of spores per cc. in the inoculum. These relations are expressed mathematically.

* This report is one of four, to be numbered 4(I), 4(II), 4(III), and 4(IV). The four reports will comprise the annual report on this project.

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Physiological studies on spore germination with special reference to Clostridium botulinum.*

II. Quantitative aspects of the germination process.

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Though virtually every aspect of the growth and death rates of bacterial cultures has been subjected to searching kinetic analysis, the spore germination process itself has been largely neglected. Probably this is due particularly to the failure to discriminate precisely between the actual germination process and the subsequent vegetative development and, especially with anaerobes, to inadequate counting methods. The only two reports really bearing on this issue, both dealing with aerobic spore formers, failed to evaluate the process other than to conclude that numbers of spores germinating increased with time (Fischoeder, 1909; Eckelmann, 1918). This paper deals quantitatively with the germination process in the anaerobic spore former Clostridium botulinum strain 62A.

Experimental

The germination medium was Difco brain heart infusion broth with BBL thioglycollate supplement. The counting medium, procedures and other details are as described in the preceding paper (Wynne and Foster, 1948).

It was observed repeatedly that the logarithm of numbers of residual spores in a spore suspension in a germination medium plotted against time gives a straight line, at least until 95 percent or more of the originally present spores

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have germinated.

Data for a typical experiment are given in Table 1, and they are plotted as Curve A in Fig. 1 together with Curves B and C, the latter two representing experiments in which the germination was allowed to take place in air, as contrasted to the natural gas atmosphere (CH_4) in the experiment described by Curve A and Table 1. The general equation for a first order reaction may be written as

$$.434K = 1/t \log \frac{I}{I - G} \qquad (1)$$

where K = a constant
t = time elapsing since beginning of germination (t_o)
I = no. spores per cc. at beginning of germination
G = germinated spores at time t

But since I - G = R (residual spores), we may substitute R in the equation above giving,

$$.434K = 1/t \log I/R \qquad (2)$$

or

$$K = \frac{(\log I - \log R)}{.434t} \qquad (3)$$

The I values in Table 1 were computed assuming t_o = 20 hours, which obviously is a minimum value, as under the experimental conditions t_o could have been any time between 20 and 22 hours. Owing to this experimental inaccuracy the K value for the 22 hour period is off, but is reasonably constant for the other periods, indicating that the germination process conforms to a first order reaction. Curve A, Fig. 1, shows also that the germination process is logarithmic, thus conforming to the kinetic picture typical of all growth and killing rates of bacteria.

It is obvious from Fig. 1 (Curves B and C) that the duration of the lag period depends on the concentration of the inoculum and is inversely proportional to it. By extrapolating the logarithmic germination curves B and C in Fig. 1, as well as others, values representing the length of the lag period were obtained according to the inoculum density as given in Table 2. The length of the lag period appears to

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vary as the reciprocal of the logarithm of inoculum numbers, and obeys the following relation:

$$L = \frac{C}{\log I} \qquad (4)$$

where L = length of lag phase in hours
I = no. of spores per cc. in inoculum
C = a constant.

The validity of this expression is borne out by the fact that the values of $\frac{I}{\log I}$ plotted against L made a straight line (Fig. 2); computed values for C at the different levels of inoculum agree fairly well, as seen in Table 2.

These data were obtained from cultures incubated in ordinary air. Curiously, these relations did not apply when incubation was done in a desiccator with an atmosphere of natural gas, at least under these conditions.

Discussion

Neither Fischoeder (1909) nor Eckelmann (1918) drew any conclusions pertaining to the kinetics of the germination observed by them, but a plot of their data against time shows they actually were concerned with logarithmic germination processes. However, data of the former author for germination of *Bacillus anthracis* in goat and dog sera indicate that germination was not logarithmic under those conditions. It is obvious that any factor inimical to germination may at once rule out the logarithmic relation.

The tentative equation suggested above for the length of the lag period obviously should be tested further with more data. If this equation is valid, plotting the length of the lag phase against logarithm of inoculum directly should give a hyperbola, since (L)(log I) = C. Though such a plot from the values determined above is compatible with a hyperbolic curve, the points are too few to define the curve clearly.

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In contrast to the paucity of information on the lag period of spore germination an abundance of work has been done on vegetative cells, but as spore germination does not involve actual cell multiplication we consider the spore problem one distinct from vegetative activity. The apparent failure of equation (4) to hold in an atmosphere of natural gas (i.e. no oxygen) cannot be explained at present. Suspicion might be directed to influence of the O-R potential on germination, an effect found to apply to spores of the anaerobe Bacillus tetani (Fildes, 1929; Knight and Fildes, 1930). Rapidity of germination depended on the time required for the medium, and presumably the interior of the spores, to reach a suitable reducing intensity, and spore numbers might influence this action. Finally, natural gases may contain traces of impurities which might account for this discrepancy.

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Wynne, E. Staten, and Foster, Jackson W. 1948 Physiological studies on spore germination with special reference to Clostridium botulinum. I. Development of a quantitative method. J. Bact. In press.

Table 1

Germination in Natural Gas

Incubation hours	Average Count Residual Spores	Germinated Spores	% Germination	Log Residual Spores	K calculated for t_o=20 hrs
0	560	-	-	2.75	
20	560	0	0	2.75	
22	230	330	59	2.36	.449
24	200	360	64	2.30	.259
*28	74	485	87	1.87	.254
30	39	520	93	1.59	.267
32	28	530	95	1.45	.250

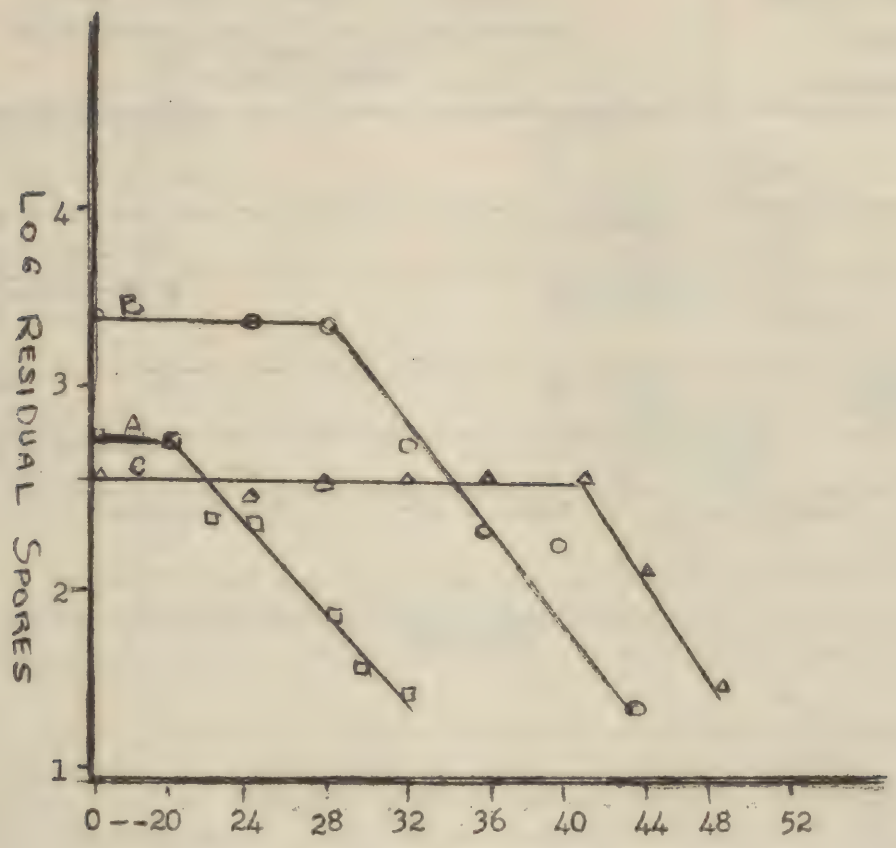
* Counts for 26 hours are not given due to accidental overheating of the tubes in the incubator.

Table 2

Relation of Duration of Lag to Concentration of Inoculum in Air

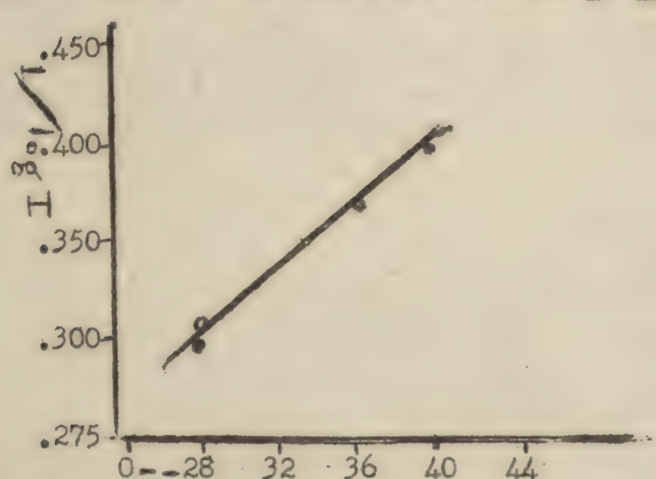
Inoculum, Spores/cc	Lag Period, Hours	C, calculated *
2400	28	95
2100	28	93
530	36	98
340	40	101

* Equation (4)



Curve A: Natural gas atmosphere.
Curves B and C: Air Atmosphere

FIG. 1. GERMINATION CURVES OF C. BOTULINUM 62A



LENGTH OF LAG PERIOD IN HOURS

FIG. 2. RELATIONSHIP OF LENGTH OF LAG PHASE OF GERMINATION TO RECIPROCAL OF LOG OF NO. OF SPORES PER CC. IN INOCULUM

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COOPERATING INSTITUTION University of Texas	LOCALITY Austin, Texas	
DIVISION Arts and Sciences	DEPARTMENT Bacteriology	
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TITLE: [ ] PROGRESS REPORT [x] PHASE REPORT [ ] ANNUAL REPORT [ ] TERMINATION REPORT

Spore Formation and Spore Germination of Anaerobic Food Spoilage Organisms; Especially Clostridium botulinum

SUMMARY

Carbon dioxide has been shown to be essential for germination of the spores of Clostridium botulinum 62 A in a synthetic medium. CO_2 could not be replaced by a mixture of malate, fumarate, succinate, α-ketoglutarate, glutarate, aspartate, glutamate and cis-aconitate. 1% yeast extract was found to replace CO_2 qualitatively, at least. It is concluded that hitherto unrecognized substances of unknown nature are necessary for bypassing the CO_2 requirement of spore germination in this organism.

In a complex medium CO_2 was only stimulatory, and could be replaced completely by oxalacetate, as well as partially by a mixture of the stable C_4 dicarboxylic acids. CO_2 could not be shown to exert any effect on germination of the spores of 4 other anaerobes or 4 aerobic species in complex media, but germination in one of the latter was significantly stimulated by the stable C_4 dicarboxylic acids.

Continued

Physiological studies on spore germination, with special reference to Clostridium botulinum*

III. Carbon dioxide and germination. Also a note on CO_2 and aerobic spores

E. Staten Wynne and Jackson W. Foster

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The requirement of CO_2 for vegetative cell development of bacteria is common knowledge and needs no review here, but scarcely anything is known of the relation of CO_2 to the process of bacterial spore germination as considered distinct from subsequent vegetative development. One might consider the latter in the sense of transition from heat stable to heat labile form. (Wynne & Foster, 1948a). Relevant is the incidental observation that spores of one out of 3 strains of C. botulinum failed to produce colonies in 72 hours when incubated in a vacuum (Morrison & Rettger, 1930).

The experience is a rather common one that special efforts to eliminate CO_2 from the culture system, and to minimize the formation of CO_2 by the cells in the inoculum by supplying a low nutrition level medium result in a retardation of growth which may extend indefinitely.

Our study of factors determinant in the germination process itself (as distinct from subsequent vegetative development) of Clostridium botulinum begun in two previous papers (Wynne & Foster, 1948a and b) has included examination of the CO_2 effect. This stems from the finding that anaerobiosis secured by alkaline pyrogallol seems to delay germination of "bot" spores. Background information and general methodology are covered in the first of these papers and need not be reiterated here. To secure anaerobic conditions free of CO_2, vacuum desiccators containing the culture tubes were evacuated with a Cenco Hyvac pump and refilled with natural (illuminating) gas (CH_4) cleansed of CO_2 by slow passage through a gas washing train consisting of 3 bottles of NaOH and one of N/10 $Ba(OH)_2$. The latter was second last in the chain, functioning as a CO_2 indicator. As an added precaution normal NaOH was always placed in the bottom of the desiccator. Where a CO_2 atmosphere was required, it was added from a cylinder or generated in the desiccator by mixing excess acid with the calculated amount of solid $NaHCO_3$. Unless otherwise specified germination always took place in Difco brain-heart infusion broth with BBL thioglycollate supplement, and always the inoculum was about 500 spores per ml. of medium. Table 1 compares the spore germination in atmospheres containing 0, 1 and 5 percent CO_2 respectively. The CO_2 effect is striking. Germination is negligible in the absence of CO_2, whereas almost all the spores germinated in the presence of CO_2, the higher CO_2 tension being somewhat better. The difference between the two CO_2 treatments actually was greater than it appears; turbidity developed in 15 hours in the 5% CO_2 desiccator and in 19 hours in 1% CO_2. No turbidity appeared in the zero CO_2 control

*This project has been undertaken in cooperation with the Committee on Food Research of the Quartermaster Food and Container Institute for the Armed Forces. The opinions or conclusions contained in this report are those of the authors. They are not to be construed as necessarily reflecting the views or endorsement of the War Department.

at 22 hours, the termination of the experiment.

However, a CO_2 effect could not be obtained for four other species of anaerobic spore formers tested similarly: Clostridium chauvei, C. histolyticum, C. perfringens, and the well known food spoilage organism designated as putrefactive No. 3679. This was true even at pH 6.0, chosen to reduce the solubility of CO_2 in the medium and being the lowest pH supporting germination of these anaerobes. Thus under identical conditions germination of C. botulinum spores is inhibited by lack of CO_2, and germination of the other four anaerobes is not. A possible interpretation of this is given in the discussion below.

The experiment described above (also Table 1) demonstrates only a rate effect under the conditions used, for whereas only 7 percent germination occurred in the CO_2 free control at the 22 hour period, prolongation of incubation always resulted in high germination and pronounced turbidity. Failure to demonstrate an absolute CO_2 effect in brain-heart medium, even with several painstaking experiments involving modification of pH, exhaustive pumping, omission of the colloidal agar of the anaerobic supplement, continuous gassing with N_2, etc., was considered on two counts as being most likely tied up with the complex nature of the brain-heart medium: (1) the CO_2 effect can be accentuated by eliminating complex media in favor of synthetic (Gladstone, Fildes and Richardson, 1935) or employing complex media at a minimal nutritional concentration, i.e., with respect to carbohydrate and protein content (Rockwell and Highberger, 1926 and 1927). Count (1) may really be considered to anticipate the issue of count (2) which states that CO_2 should be dispensable so long as certain organic substances are present in whose synthesis CO_2 participates. The presence of such substances is likely in complex media of biological origin and in such media therefore the need for CO_2 should be obviated. While our attack on these lines was under way other reports have appeared which confirm the logic of this approach. (C_4 dicarboxylic acids, White and Werkman, 1947; aspartic acid, Lardy, et al, 1947 and Lardy, 1947.)

A synthetic medium similar to that devised by Roessler[1] (1946) for growth of C. botulinum was used as a starting point, but with only 1/10 the regular concentration of amino acids. (Results were similar, however, with the full medium, which contains 1% amino acids.) This media supported abundant vegetative development of our strain of C. botulinum and spore germination was much slower than in complex media, seemingly opening an approach to factors essential for germination, including those involving CO_2 and not. For good anaerobic growth it was expeditious to add 0.2% glucose to the synthetic media, as germination is negligible in its absence.

With an inoculum of around 500 spores per ml., clear cut turbidity developed in the presence of CO_2 at about 72 hours but counts at 87 hours showed that only about 15% of the spores had germinated. In subsequent work a 5-day incubation period was employed for positive CO_2 controls, for in this time germination counts were well over 50 percent. In such a medium it is possible to approach an absolute CO_2 requirement for germination. Thus in an experiment

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1 This medium has the following composition: dl-leucine, .0083M; dl-phenylalanine, .0132M; l-arginine, .0065M; dl-valine, .0083M; dl-isoleucine, .004M; l-tryptophane, 0.11M; l-tyrosine, .0003M; dl-methionine, .002M; dl-threonine, .0067M; dl-serine, .01M; l-histidine, .0013M; biotin, 5 mγ/cc; PABA, .02 γ/cc; nicotinamide, 1 γ/cc; thiamin, 0.2 γ/cc; yeast nucleic acid, .01%; Na thioglycollate .05%; $MgSO_4$, .0002M; $MnSO_4$, .0001M $CaCl_2$, .0001M; $FeSO_4$, .00005M; K_2HPO_4, .015M; KH_2PO_4, .015M.

where the positive (1%) CO_2 control showed 61% germination in 5 days, the CO_2 free treatment showed only 11% germination and no turbidity even after 17 days (Table 2). Indeed, the figure of 11% may not be significant at all owing to the fact that the spore counting method has an overall accuracy of ± 9% and occasionally with spreads wider than this. (Wynne & Foster, 1948a). Under these conditions clear cut turbidity always follows germination within a few hours. Thus probably no germination at all occurred in the CO_2 free tube, the 11% value doubtless being an experimental counting error.

It seems safe, therefore, to conclude that CO_2 is absolutely essential for spore germination of C. botulinum in a medium otherwise adequate for that process. This apparently is the first demonstration of CO_2 requirement specifically for the germination process, and apart from subsequent vegetative development.

By-passing CO_2.

Oxalacetic acid -- Along the lines discussed in count (2) above the C_4 dicarboxylic acids were tested for their ability to permit germination in the absence of CO_2, as the universality of the Wood-Werkman reaction via pyruvate fixation of CO_2 indicates the likelihood of their being involved here. The primary fixation product, oxalacetic acid (OAA), is generally in biological equilibrium with malic, fumaric and succinic acids, all vital catalysts or intermediates in cells. OAA in brain-heart media definitely promotes the germination rate of bot spores in the absence of gaseous CO_2 (See Table 3) and apparently bypasses CO_2. Experiment A in Table 3 shows that the OAA induced spore germination at a rate appreciably faster than a 1% CO_2 gas tension, and in Experiment B it was equal to the CO_2 in promoting germination. In experiment B the chances are that OAA also would have shown up a faster germination had the counts been made at a shorter incubation period.

The OAA effect might, to a certain extent, be ascribed to CO_2 resulting from the spontaneous decomposition of OAA to CO_2 and pyruvic acid (Krampitz and Werkman, 1941; Krebs, 1942). OAA in solution at 37° has a very short half life and its decomposition is catalyzed by amino groups and by traces of cationic metals. However, since OAA gives a germination rate exceeding that of CO_2 the effect seemingly is due to the OAA per se, though CO_2 may contribute to the rate partially. Germination by OAA was not retarded when the medium was continuously exhausted with a Hyvac pump for 4.5 hours after OAA addition, the idea being to remove quickly any CO_2 generated from OAA. (Exp. B, Table 3). As no lessening of the OAA effect by this continuous CO_2 removal was observed the probability of a direct OAA participation seems good.

Maybe a brief contact with CO_2, such as would occur in the above pumping experiment, would suffice for germination, but other experiments showed that contact with a 1% CO_2 atmosphere for the initial 4 hours followed by removal (Hyvac) and replacement with CO_2 free gas had an insignificant effect on germination.

Stable C_4 dicarboxylic acids.-- A mixture of l-malic, fumaric and succinic acids (Na salts), each at a concentration of 3.3×10^{-4} M was shown repeatedly to have a definite acceleration on germination rate in the absence of CO_2. These acids were not as effective as CO_2 (or OAA) in promoting germination. The efficacy of these acids in promoting spore germination was roughly about 1/3 that of a 1% CO_2 gas phase. The inability of the acids to substitute fully for OAA has been encountered previously (Shive and Rogers, 1947, and others) and probably relates to membrane penetration at pH values in physiological range, in which these acids

are almost 100 percent dissociated. It will be recalled that OAA itself does not penetrate unaltered cells of Micrococcus lysodeikticus (Krampitz and Werkmann, 1941), and several other examples could be given. If these acids diffuse in the molecular (undissociated) form as the free acids, it would be expected that diffusion would be greatest at pH 3 to 4 as the acids are almost entirely in molecular form in this range as contrasted to a negligible percentage at pH 6 or above. It was not possible to test this with C. botulinum as germination is inhibited at pH values below 6. It will be recalled that White and Werkman (1947) found that the C_4 dicarboxylic acids or their respiratory precursors bypassed the CO_2 requirements for coliform bacteria.

The specificity of the effect for the C_4 dicarboxylic acids on bot germination is exemplified by the fact that no demonstrable action was given by α-ketoglutarate, glutarate, valerate, butyrate, propionate, lactate or pyruvate. On the other hand a striking stimulation in vegetative development was induced by all of these acids (except pyruvic) at 10^{-3} M. So marked was this that cultures with well advanced turbidities showed surprisingly small germination percentages. This is a fine example of the fallacy of judging germination rates by the intensity of vegetative turbidity.

Aspartic acid. -- As OAA is converted to aspartic acid by transamination, one might expect this amino acid also would bypass the CO_2 requirement, the latter participating in the synthesis of aspartate. This has indeed been demonstrated for Lactobacillus arabinosis (Lardy et al, 1947; and Lardy, 1947) in which case aspartate is apparently the only constituent of cell material in whose synthesis CO_2 participates, excepting perhaps for relatively insignificant amounts of other components. This was proved by isotopic CO_2, substantially the entire content of the labelled C in the cells being in the carboxyl groups of the cellular aspartate. It is likely that the other C_4 dicarboxylic acids are converted to aspartate via OAA.

The bot germination test was conducted in Koessler's synthetic medium (1/10 strength amino acids) which, as a basal medium, lacked $NaHCO_3$, biotin, and aspartic acid. The following treatments were set up in triplicate tubes of the basal medium:

(1) CO_2 free gas phase (zero control)
(2) 1% CO_2 gas phase
(3) 5 mγ biotin/ml in CO_2 free gas phase
(4) 5 mγ biotin/ ml. in 1% CO_2 gas phase
(5) 10^{-3} and 10^{-4}M aspartate respectively in CO_2 free gas phase
(6) 10^{-3} and 10^{-4}M aspartate respectively plus 5 mγ biotin/ml. in CO_2 free gas phase.
(7) 10^{-3} M and 10^{-4} M α-ketoglutarate respectively plus 5 mγ biotin/ml. in CO_2 free gas phase
(8) 10^{-3} M aspartate plus 1 and 10 mγ oleate/ml. respectively in CO_2 free gas phase

Oleate was tested because of its known biotin sparing action, and α-ketoglutarate because conceivably it could generate C_4 dicarboxylic acid precursors of aspartate. After 4 to 5 days incubation, turbidity commenced in only treatments (2) and (4), namely those with CO_2 in the gas phase. Spore counts made of these on the 7th day showed 60% germination. The remaining 6 treatments showed no turbidity up to the 17th day, when they were removed for spore counts. In every case spore germination was nil or negligible and it may be concluded that the substances used, and in the combination tested, could not by-pass the requirement for CO_2.

In the next experiment biotin, aspartate, oleate were tested all in one mixture, in the concentration ranges as before, except that 100 m γ/cc of oleate was also tried, and again germination took place only in those treatments with a CO_2 containing gas phase, both with and without the test supplements, whereas germination was insignificant in the absence of CO_2 even after prolonged incubation (14 days).

Finally, the following known or suspected bypassing substances and available participants in the tricarboxylic acid respiratory system were tested in combination, all at 10^{-3}M in basal synthetic medium, in the presence and in the absence of CO_2: aspartate, malate, fumarate, succinate, α-ketoglutarate, glutamic acid, glutarate, and cis-aconitate. These were entirely unsuccessful in bypassing CO_2. When CO_2 was present, the germination rate was unaffected in this medium, indicating no toxicity caused by the supplements.

Complex Supplements -- Also, the following complex organic supplements were tested in triplicate tubes at 0.1 and 1.0% levels in the basal synthetic medium, again in the absence and in the presence of CO_2: brain heart infusion, liver extract[2] and yeast extract, all Difco. The CO_2 free yeast and liver treatments were incubated in one desiccator, the CO_2 free brain-heart in another, and the CO_2 free synthetic medium in another. Within 40 hours in the absence of CO_2 all the yeast tubes developed marked turbidity and a single 1% liver and a single 1% brain heart. To avoid contaminating the other tubes with fermentation CO_2, these turbid tubes were removed, pasteurized and held for spore counts. All the tubes of synthetic medium in CO_2 showed turbidity at 3 to 4 days and were removed for counting on the 5th day. The CO_2 free synthetic medium showed no turbidity even after 15 days, the termination of the experiment, and the remaining liver and brain-heart tubes in CO_2 free atmosphere behaved similarly. Residual spore counts for this experiment are in Table 4.

It is clear that yeast contains CO_2 bypassing factor(s) which are not identical with the supplements added to the basal medium, because CO_2 was necessary for germination in the latter treatment, but not in the yeast. Yeast apparently is richest in the unknown bypassing factor(s) as the liver and the brain-heart were greatly less effective in this respect. The rapid growth in the yeast tubes in the same desiccator as the negative liver tubes shows the effect resides specifically in their contents of CO_2 bypassing substances and not a CO_2 leak or other artifact leading to the unintentional presence of CO_2, for the smallest amounts of CO_2 induce rapid germination in the liver (and brain-heart medium). One will note that even the amounts of CO_2 generated by the turbid yeast tubes were insufficient to induce significant germination in brain heart medium.

Aerobic Spore formers.

Some testing of a survey nature was done with four species of aerobic spore formers, *Bacillus brevis*, *B. megatherium*, *B. mesentericus* and *B. subtilis*. Germination was done in Difco nutrient broth in shallow layers in 50 cc. Erlenmeyer flasks at room temperature. CO_2 free treatments were conducted in desiccators with air as the gas phase. In no case was it possible to retard germination of these organisms in a CO_2 free atmosphere. Evidently this is due to the presence in the nutrient broth of organic substances bypassing the CO_2, although no attempt was made to confirm this with synthetic media. Interestingly enough, though CO_2 did not enhance the germination of any of these four aerobes, in one, *B. mesentericus*, the stable C_4 dicarboxylic acids mixture (3.3×10^{-4} M each)

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2 Extract of 0.1 and 1.0% dried liver

distinctly accelerated the germination. Thus in a typical experiment with an inoculum of 3040 spores per ml., 29 percent germination was obtained in the CO_2 free treatment after 25 hours, and 74 percent in the C_4 treatment. This organism presumably was inefficient in the conversion of CO_2 to C_4 dicarboxylic acids.

The behavior of vegetative development of each of these aerobes in relation to CO_2 is in decided contrast to the spores, for in each case CO_2 induced a marked acceleration. This again emphasizes the distinction between the germination process and subsequent vegetative activity of sporeforming bacteria.

Discussion and Summary

The germination process and vegetative cells are not affected alike by CO_2 and the C_4 dicarboxylic acids. The fact that germination in four out of five anaerobes tested failed to respond to CO_2, whereas C. botulinum did, indicates species or strain differences. This applies also to the differences described for the four aerobic spore formers.

A clue to the nature of these effects comes from the fact that in complex media (i.e., brain-heart infusion) CO_2 deprivation only slowed down the rate of germination but did not stop it, whereas in synthetic medium germination could be entirely suppressed indefinitely without CO_2. Judging from the evidence available, this could mean that present in complex media are substances as yet unknown which can bypass CO_2. Some such substances are known (vide supra, C_4 dicarboxylic acids and aspartic acid) but these could not substitute for CO_2 in the germination of bot spores in a synthetic medium which is otherwise adequate for germination and growth. Does this mean, then, that present in complex natural materials are additional new substances capable of bypassing the CO_2 requirement, and in whose synthesis CO_2 participates when they are not supplied artificially to the medium? Seemingly the positive germination results obtained in a CO_2 free system upon addition of small amounts of yeast extract to the basal synthetic medium, speak in this behalf. After this work was completed the recent report of Lwoff and Monod (1947) was received. These authors, working with E. coli, found C_4 and C_5 dicarboxylic acids and their amino derivatives to be effective CO_2 bypassing agents, but that they alone did not suffice, and they came to exactly the same conclusions as above: namely, other essential CO_2 bypassing agents are present in complex natural materials.

One may easily visualize that CO_2 is involved in synthesis of biological substances other than C_4 and C_5 acids and the derived aspartic and glutamic acids, and, indeed, at least one other system already is known, viz., carboxylation of α-ketoglutaric acid to oxalosuccinic acid (Ochoa, 1945), others are under suspicion, and new ones not only are a distinct possibility but on the basis of the above evidence must exist.

Variations in response to C_4 dicarboxylic acids mean that these are required to different degrees by different organisms. Thus, the response by C. botulinum in the complex medium to added C_4 acids indicates these were limiting or near limiting in germination. Complete lack of response to CO_2 by the other Clostridia indicates that whatever bypassing agents (presumably including the C_4 acids) were present were sufficient to bypass CO_2 entirely. Similar differences showed up in the aerobes: though removal of CO_2 did not retard germination in any of the four species, C_4 acids significantly stimulated germination in B. mesentericus and were therefore limiting even in the presence of CO_2. It is possible that the C_4 acids may fully bypass CO_2 in this organism. If C_4 acids play a role in the by-

passing of CO_2 in the other three aerobes, the concentration present in nutrient broth must be adequate, though other substances may be involved.

The main conclusion obtainable from all these observations is that organisms differ widely in the extent to which medium components enable them to bypass their CO_2 requirements and that some hitherto unrecognized CO_2 bypassing substances exist. A corollary is that a complete diet of organic compounds renders CO_2 dispensible for germination and initiation of growth.

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Table 1

EFFECT OF CO_2 CONCENTRATION

ON GERMINATION

Incubation Hrs.	CO_2 Tension %	Residual Spores	Germinated Spores	% Germination
0	---	560	---	---
22	0	520	40	7
22	1	90	470	84
22	5	18	540	97

Table 2

Quantitative indefinite inhibition of germination of _C. botulinum_ spores due to the absence of CO_2.

CO_2 in atmosphere, %	Incubation days	Av. count residual spores	Germinated spores	% Germination
-	0	560	-	-
*0	17	500	60	11
1	5	220	340	61

* Counts corrected for volume loss of 9% during prolonged incubation over NaOH. No turbidity developed in any of the tubes in this series.

Table 3

Effect of oxalacetate on germination in CO_2-free gas phase

Experiment A

Incubation, hours	CO_2 in atmosphere, %	O.A.A., 10^{-3} M	Av. count residual spores	Germinated spores	% Germination
0	-	-	535	-	-
20	0	-	460	75	14
20	1	-	340	195	36
20	0	+	74	460	86

Experiment B

Incubation, hours	CO_2 in atmosphere, %	Oxalacetate added	Exhaustion period, minutes	Av. Count residual spores	Germinated Spores	% Germination
0	-	-	-	520	-	-
23	0	0	30	470	50	10
23	0	10^{-3} M	30	35	485	93
23	0	10^{-3} M	270	65	455	88
23	1	0	30	21	500	96

Table 4

Effect of Complex Supplements on Germination

Incubation Days	% CO_2	Supplement Added	Avg. Count Residual Spores	Germinated Spores	% Germination
0			450		
1	+	1% Yeast extract	15	435	97
1	+	1% Liver extract	230	220	49
1	+	1% Brain heart	35	415	92
2	-	1% Yeast extract	45	405	90
2	-	0.1% Yeast extract	120	330	73
2	-	1% Liver extract*	385	65	14
2	-	1% Brain heart*	50	400	89
5	+	None	160	290	64
15	-	None	425	25	5
15	-	1% Liver extract†	475	0	0
15	-	0.1% Liver extract	475	0	0
15	-	1% Brain heart†	430	20	4
15	-	0.1% Brain heart	410	40	9

* One out of three tubes of which † represents remaining two.

Doc
W2
A2
qQ12p

PROJECT REPORT COMMITTEE ON FOOD RESEARCH QUARTERMASTER FOOD AND CONTAINER INSTITUTE FOR THE ARMED FORCES CHICAGO ILLINOIS		RESEARCH AND DEVELOPMENT BRANCH MILITARY PLANNING DIVISION OFFICE OF THE QUARTERMASTER GENERAL
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DIVISION Arts and Sciences	DEPARTMENT Bacteriology	
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REPORT NO. 4 (IV)	FILE NO. M-605	CONTRACT NO. W11-009-qm-70190
FOR PERIOD COVERING August 1946 - April 1947	INITIATION DATE 1 July 1946	

TITLE: [ ] PROGRESS REPORT [ ] PHASE REPORT [X] ANNUAL REPORT [ ] TERMINATION REPORT

Spore Formation and Spore Germination of Anaerobic Food Spoilage Organisms, Especially Clostridium Botulinum

SUMMARY

The effects of concentration of medium, salts, pH, surface tension, temperature, visible light, and oxygen on sporulation in C. botulinum have been studied. Over the concentration ranges tested, an exponential relationship appeared to exist between the molar concentration of the salts used (NaCl, KCl, and Na_2SO_4) and total spores or percentage spores resulting.

Physiological Studies on Spore Formation in Clostridium botulinum*

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The voluminous literature on the physiology of spore formation in bacteria has been reviewed b several workers, including Dozier (1924), Brunstetter and Magoon (1932), Cook (1932), and Knaysi (1945). Comparatively little work has been done on the sporulation process in Clostridium botulinum, the most extensive being probably that of Leifson (1931). As part of a larger program on spores of C. botulinum in relation to the importance of this organism as a hazard in foods a systematic study has been made of factors influential in formation of its spores.

Methods

Cultures

The strains of C. botulinum were with one exception (Texas No. 29) obtained from the National Canners' Association. Strain 62A was employed for most of the work. The others were numbered 78, 6B, 116B and 213B. Toxicity was demonstrated for 62A by the fact that 1.0 ml of a Seitz filtrate of a 10-day broth culture was lethal for a guinea pig in less than 21 hours, while a control animal receiving 1 ml of the filtrate inactivated at 80 C for 15 minutes survived the observation period of 3 weeks.

Medium

Comparison of spore formation by all six strains above in veal infusion peptone, liver infusion peptone, liver-liver infusion, BBL fluid thioglycolate and Difco brain heart infusion, resulted in the choice of brain heart infusion broth with BBL thioglycolate supplement as a basal medium for these studies. Ordinarily no anaerobic device was needed, as deep tubes of the medium, preferably boiled just prior to inoculation, gave excellent growth from loop inocula in less than 24 hours. Sporulation was not significant for any strain in the synthetic medium of Roessler (Wynne and Foster, 1948) with 0.1 percent agar added to improve anaerobiosis.

Staining and Microscopic Counting

Comparison of several spore staining methods resulted in the adoption of the following procedure, which represen s a combination of May's (1926) use of a chromic acid mordant with Conklin's (1934) stain:

1. Treat heat-fixed films with 5 percent chromic acid for two minutes. Wash.
2. Cover with 5 percent aqueous malachite green and steam 5 minutes.
3. Destain with H_2O 5 to 10 seconds.
4. Counterstain with 5 percent aqueous mercurochrome 20 to 30 seconds. Wash.

This method has consistently given beautiful preparations readily allowing quantitative differentiation of spores and vegetative cells. Clostridial forms

* This project has been undertaken in cooperation with the Committee on Food Research of the Quartermaster Food and Container Institute for the Armed Forces. The opinions or conclusions contained in this report are those of the author. They are not to be construed as necessarily reflecting the views or endorsement of the War Department.

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retaining the green dye in the primordium have arbitrarily been considered spores. Forms taking the pink counterstain with no green bodies within have been counted as vegetative cells. The total of spores and vegetative cells counted for each determination was at least 200 or more. These figures permitted computation of percentage of spores out of the total population.

Total spore and vegetative cell count per ml of broth was determined in the Petroff-Hauser chamber. Staining was unnecessary for this purpose as the refractile spores are easily distinguished with the 43X lens and subdued illumination. Duplicate or triplicate counts were ordinarily made on each of triplicate tubes for each determination, with 50 to 200 small squares counted, representing a total of 200 or more cells per count. Series of five counts each on two cultures showed the deviation from the mean to be around ±12 percent. Good correlation was consistently obtained between percent spores obtained from Petroff-Hauser counts and the staining method above. For example, an average of four determinations on a culture by the Petroff-Hauser method gave 16.8 percent spores, as compared to 13.8 percent by stained smears. The staining method is superior when the percentage of spores is low, and it was routinely used to check results obtained by the Petroff-Hauser counts where the spore percentage was 10 or less. It should be emphasized that zero spores in the tables simply means too few to detect by staining, i.e., less than 0.5 percent.

Effect of various environmental factors on sporulation

Concentration of Medium and Medium Components

Conflicting reports exist in the literature dealing with the effect of concentrated media versus dilute media on rate and relative amount of sporulation with organisms in the genus Bacillus. The more recent studies seem to indicate that percentage of spores is increased in more dilute media. This particular factor appears not to have been studies in C. botulinum.

Concentrations of brain heart infusion broth (Difco) from 1/4 to 5 times the normal strength have been tested, each with the usual BBL supplement added. A striking inhibition of sporulation occurred in concentrations above that normally used. (Table 1). This applied not only to total number of spores but percentage of spores. This depression of sporulation occurred despite the fact that in the 2X and 4X medium strengths the total cell counts were not strikingly different from the count in the normal (1X) medium. The percentage of spores was not changed appreciably in the X/2 and X/4 media though the total counts were reduced.

The ingredients of the medium were tested individually by addition to regular strength broth so that the final concentration of the component was 5X that in normal broth. Four percent "Bacto" peptone was also tested since it has been reported that Bacto-peptone, but not proteose-peptone, contains a factor inhibiting sporulation in B. subtilis (Roberts and Balwin, 1942). Of all the individual substances tested (Table 1) NaCl was the only one which specifically diminished. spore formation, without any significant effect on the vegetative population. Other experiments have shown, in fact, that 2 percent added NaCl generally gives less than 0.5 percent spores. The failure of 1 percent added Na_2HPO_4 to depress percent spores (though it did decrease total spores somewhat) is not surprising in view of Leifson's (1931) finding that the phosphate ion stimulates sporulation in C. botulinum.

It is tempting to postulate the presence of a factor in the untested "infusion" portion of the brain heart medium which was responsible for the absence of detectable spores in the two most concentrated media. The slowness with which turbidity developed indicates toxicity of some sort. While visible growth occurred overnight in the control medium plus 2 percent NaCl, it developed only after 2 days with quadruple, and 3 days with quintuple, strength medium. The existence of a sporulation-inhibiting factor is rendered plausble by the reduction of about 70 percent in both total and percentage spores in double strength medium. Even more drastic reduction (over 90 percent) has been observed in double strength medium, while other experiments have repeatedly shown added 0.5 percent NaCl to effect a reduction in total spores of only 25 to 30 percent, with still less effect on spore percentage.

The failure of 0.8 percent added glucose to affect sporulation significantly may seem surprising, particularly since the final pH was reduced to 6.4. Relevant is the observation by Matzuchita (1902) that 5 to 10 percent glucose was optimum for spore formation in this species.

Salts

It has been reported that proper concentrations of inorganic salts selectively inhibit sporulation in C. botulinum (Leifson, 1931). Chloride and nitrate ions were effective in inhibition, while ammonium and phosphate, and to some extent sulfate, ions stimulated spore formation. In extensive physiological studies on sporulation in certain Bacillus forms, Schreiber (1896) concluded that salts such as KNO_3, $MgSO_4$, NaCl, and K_2HPO_4 in concentrations which inhibit growth also delay the development of spores.

The effect of varying concentrations of NaCl, KCl, and Na_2SO_4 added to brain heart infusion are given in Table 2. Values for log of total spores v. log molar concentration plotted in Figure 2 seem to give a straight line in each case, considering the estimated ± 12 percent accuracy of the spore counting method used. Such would be expected if the relationship between concentration of these salts and the resulting absolute number of spores is an exponential one. Thus in the equation

$$C^n \cdot S = K \qquad (1)$$

if C = molar (or ionic) concentration
n = dilution coefficient - a constant for the particular salt used.
S = no. spores/cc
and K = a constant for the particular salt used.

Then

$$n \log C + \log S = \log K = K' \qquad (2)$$

It obviously follows that log C v. log S should give a straight line. Similar curves were obtained by plotting log C v. log percent spores.

It will be observed that although lower molar concentrations of Na_2SO_4 are more toxic to sporulation than NaCl or KCl, higher concentrations are less effective. This is a perfect illustration of Fulmer's (1925) contention that, in general, the relative action of two factors on a bacterial species cannot be determined from a study of a single equimolecular concentration, since their curves of concentration v. effect may cross, as in Figure 1.

pH

The optimum pH for spore formation in C. botulinum was found by Leifson(1931) to be 6.2 to 6.8, with both growth and sporulation ceasing at about pH 6.0. It has been suggested that fermentable carbohydrate inhibits sporulation mainly by increasing acid production (Leifson, 1931). This would be true particularly in a poorly buffered medium.

Sporulation in brain heart infusion broth with initial pH values of 6.0 to 10.0 is given in Table 3. Though spore formation practically ceased at pH 6.0, in agreement with Leifson's (1931) findings, the 70 spores/cc found by plating may well represent a significant number from the standpoint of practical food bacteriology. The point of minimum spore counts was the most favorable for maximum vegetative cell numbers. Vegetative development has been fairly good at pH values as low as 5.0.

Surface Tension

Though Larson, et al. (1925) observed a marked depression of sporulation in B. subtilis at surface tension values of less than 45 dynes/cm, this effect may well have been due to a diminution of oxygen supply, since the usual pellicle was not formed. To our knowledge no previous studies have been made on the effect of surface tension on spore formation in anaerobic bacteria. It is recognized that changes in film pressure at the medium/air interface represented by surface tension, may not necessarily parallel variations in interfacial tension at cell membrane/medium, but even approximations of the latter require rather intricate apparatus (Davis,1927).

Measurements of surface tension were made with a Du Nouy tensiometer on standard size surfaces at equilibrium. The fundamental equation for determining surface tension by this method has been represented (Harkins and Jordan, 1930) as

$$\alpha = \frac{FP}{4\pi R}$$

where α = surface tension in dynes/cm
R = mean diameter of platinum ring
P = pull in dynes (determined from scale readings on a calibrated instrument)
F = correction factor

The values of F were obtained from tables (Harkins and Jordan, 1930). Though no claim is made for absolute accuracy of α values listed in Table 4, the values of F calculated from measurements of P and R and the known values of α for benzene and H_2O checked the literature values within ± 1.4 percent.

With sodium lauryl sulfonate as the surface tension depressant, spore formation was not affected at α values above 35 dynes/cm (Table 4), but appeared to decrease logarithmically at surface tensions below this value, as seen in Figure 2.

The nature of the depressant appears more important, however, than the actual surface tension. Thus, an α value of 36 dynes/cm obtained by means of sodium lauryl sulfonate or sodium ricinoleate gave no significant depression of sporulation, while values of 36.6 and 37 dynes/cm produced by zephiran chloride and "Hyamine", respectively, completely inhibited growth. Nature of the surface

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active agents in relation to the inhibition of bacterial growth they cause has been described by several authors. (Gibbs, et al, 1926; Frobischer, 1927; Pizarro, 1927; Day and Gibbs, 1928).

Temperature

Reports on the optimum temperature for sporulation of C. botulinum show wide divergence. While spore formation was not observed above 22 C by Roemer (1900), and was claimed virtually absent above 35 C by Van Ermengem (1897), other workers have found the optimum to be 37 C (Landman, 1904; Orr, 1922).

Though our studies have not been extensive, we have observed no appreciable difference in spore formation at 30 and 37 C. However, at 24 C sporulation was depressed as much as 50 percent.

Visible Light

No effect was observed on spore formation by this factor.

Oxygen Tension

It has been reported that broth cultures of C. botulinum exposed to the air sporulate faster than those sealed with vaseline (Sommer, 1930). Traces of oxygen have also been found beneficial to spore formation in other anaerobes (Migula, 1897; Matzuchita, 1902). With B. tetani and the bacillus of symptomatic anthrax, sporulation was wholly lacking in the complete absence of oxygen. (Zinsser, 1906).

In limited studies at 13 days' incubation, we have been unable to detect differences in spore percentages between atmospheres of ordinary air and natural gas.

Discussion

Behring (1889a and 1889b) proposed the general thesis that sporulation is an intermediate stage in normal development and may be partially or completely suppressed by proper concentrations of growth-inhibiting substances, which exert a partial physiological damage to the cell short of total prevention of growth. Though Behring has not received proper credit, a tremendous amount of literature has shown the correctness of his general concept with such diverse agents as unfavorable temperature (Pasteur, et al, 1881; Phisalix, 1892; Migula, 1897; Matzuchita, 1902; Bongert, 1903-1904; Daranyi, 1930); concentration of nutrients (Matzuchita, 1902); pH (Behring, 1889a; Daranyi, 1930); pressure (Matzuchita, 1902); disinfectants (Behring, 1889a and 1889b; Roux, 1890); growth products of other organisms (Bonger, 1903-1904; Mellon, 1927); and oxygen tension (Leifson, 1931). Our own investigations are in complete accord with Behring's hypothesis.

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Table 1

Effect of Concentration of Difco Brain Heart Infusion and its Components
(BBL thioglycolate supplement present trhoughout.)

Incubation, 5 days.

Brain heart infusion	Added component, 4X	Final pH	Spores $\times 10^{-6}$	Vegetative Cells $\times 10^{-6}$	Total $\times 10^{-6}$	% Spores
5X		7.4	0	7	7	0
4X		7.2	0	37	37	0
2X		7.0	8	62	70	11
X (Control)		6.8	28	48	76	37
X/2		7.6	21	25	46	46
X/4		7.2	9	13	22	41
X	NaCl (2%)	7.0	2.5	55.5	58	4
X	Na_2HPO_4 (1%)	7.4	16	25.5	41.5	39
X	Glucose (0.8%)	6.4	26	40	66	39
X	Difco Proteose peptone (4%)	6.8	82	95	177	46
X	Difco Bacto peptone (4%)	7.6	66	156	222	30

X = normal or usual strength

Table 2

Effect of added salts on sporulation in brain heart infusion broth.

			Incubation, 11 days at 37 C			
Salt	Percent concentration	Molar concentration	Spores $x 10^{-6}$	Vegetative cells $x 10^{-6}$	Total $x 10^{-6}$	Percent Spores
---	---	---	44	46	90	49
NaCl	0.5%	.086	29.5	39.5	69	43
"	1.0	.171	2	51	53	4
"	1.5	.256	0.5	44.5	45	1
"	2.0	.342	<0.2	45	45	Negligible
"	2.5 - 5.0	.428 - .855	Good growth in 24 hours.			No spores seen.
KCl	0.5	.067	21	36	57	37
"	1.0	.134	1.5	34.5	36	4
"	1.5	.201	0.6	31	32	2
"	2.0	.268	0.3	21	21	1.5
"	2.5	.336	<0.1	21	21	Negligible
"	3 - 5	.403--.671	Good growth overnight.			No spores seen.
Na_2SO_4	0.5	.035	6.5	23.5	30	22
"	1.0	.071	4.0	22	26	15
"	1.5	.106	4.0	22	26	15
"	2.0	.141	3.0	24	27	11
"	2.5	.176	2.5	27	29.5	9
"	3.0	.211	1.8	24	26	7
"	3.5	.246	0.8	22	23	3
"	4.0	.282	1.4	12.5	14	10
"	4.5	.316	<.04	7.0	7	Negligible
"	5.0	.352	Growth delayed and scanty - - - -			

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Table 3

Effect of pH on Sporulation

Initial pH	Spores/cc $\times 10^{-6}$	Vegetative cells/cc $\times 10^{-6}$	Total/cc $\times 10^{-6}$	% Spores
6.0	.00007*	540	540	0.00001*
6.5	3.5	180	184	2
7.0	57	67	124	46
7.5	57	66	123	46
8.0	61	47	108	56
8.5	26	22	48	54
9.0	20	14	34	59
9.5	20.5	21.5	42	49
10.0	6	29	35	17

* Though no spores were detectable by microscropic examination, plating in Yesair's medium showed 70 spores/cc.

Table 4

Effect of Surface Tension on Sporulation

Concentration sodium lauryl sulfonate $\times 10^{-5}$	α, dynes/cm	Spores $\times 10^{-6}$	Vegetative Cells $\times 10^{-6}$	Total Cells $\times 10^{-6}$	% Spores
None	45.3	34	27	61	56
1	38.6	33	41	74	45
2	36.7	32	39	71	45
3	34.7	36	50	86	42
*4	34.5	16	50	66	24
+5	33.8	4	71	75	5
*6	33.2	0.3	33	33	1

* Two of three replicates grew.

\+ One of three replicates grew.

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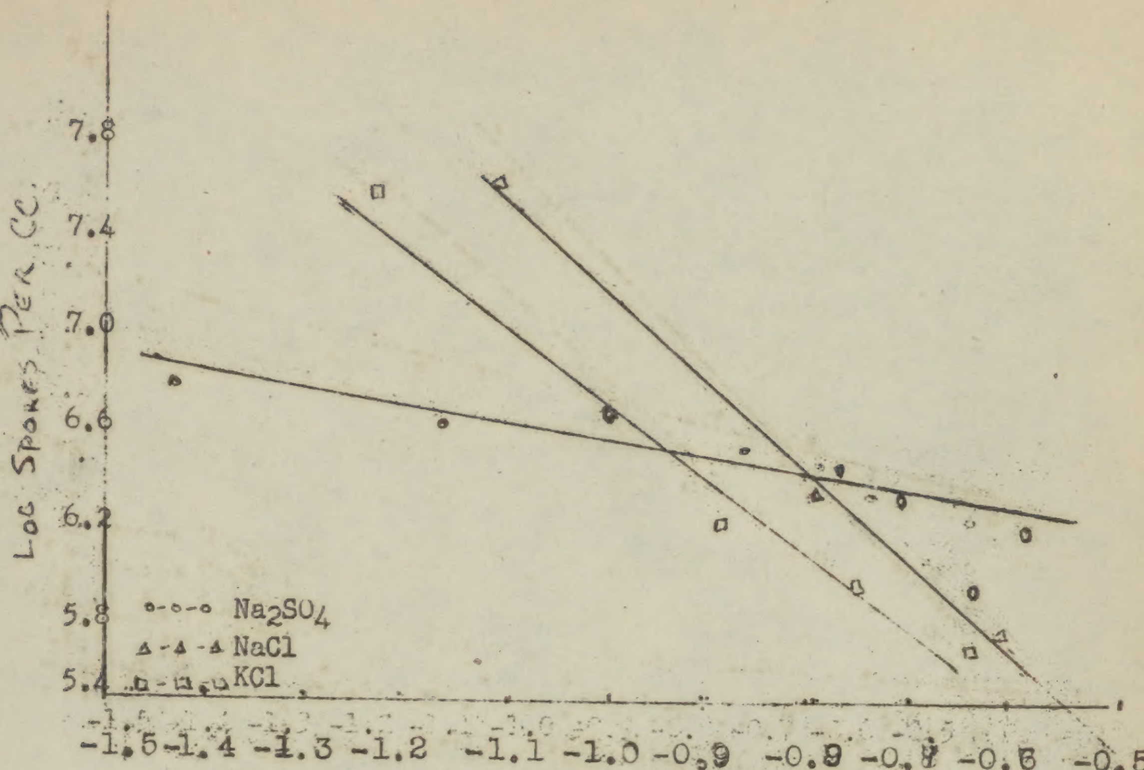
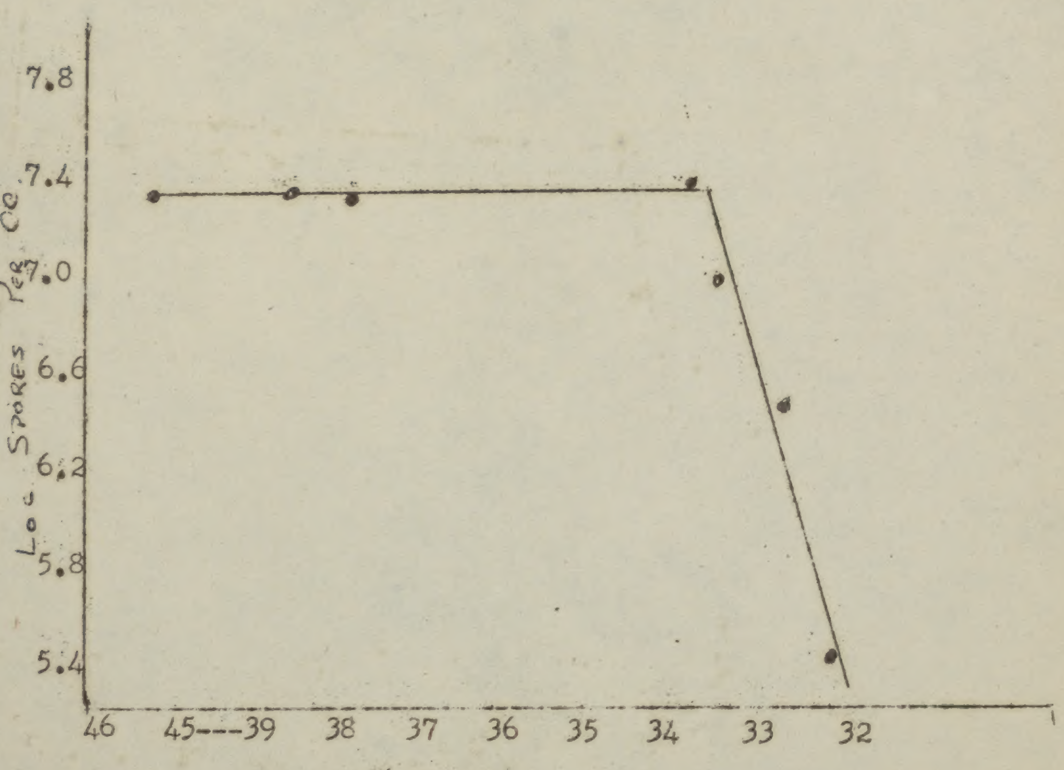


FIG. 1 EFFECT OF SALTS ON SPORULATION
OF C. BOTULINUM 62A.



α, DYNES PER CM
FIG. 2. EFFECT OF SURFACE TENSION OF SPORULATION
DEPRESSANT USED: SODIUM LAURYL SULFONATE.